PIN1 in hepatocellular carcinoma is associated with *TP53* gene status

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Abstract. Phosphorylation of proteins on serine/threonine residues that precede proline (pSer/Thr-Pro) is specifically catalyzed by the peptidyl-prolyl *cis-trans* isomerase PIN1. PIN1-mediated prolyl-isomerization induces cell cycle arrest and growth inhibition through the regulation of target proteins, including TP53. We examined whether PIN1 acts in a different manner according to *TP53* gene status in hepatocellular carcinoma (HCC). We investigated the expression of PIN1 and TP53 proteins in 119 HCC tissue samples. We also analyzed PIN1 expression in combination with *TP53* gene mutation and its correlation with the clinical outcome. In addition, we used synthetic small interfering RNA to silence *PIN1* gene expression in *TP53* wild-type and *TP53* mutant HCC cell lines, and then evaluated cell proliferation, migration and invasion. Expression of PIN1 was strongly associated with expression of TP53 protein or *TP53* mutation of HCC samples. PIN1 and TP53 expression in *TP53* mutant HCC cell lines was higher than that in *TP53* wild-type HCC cell lines. Silencing of PIN1 in HLE cells containing mutant *TP53* significantly decreased cell proliferation, migration and invasion. In contrast to PIN1 silencing in HLE cells, PIN1 silencing in HepG2 cells containing functional wild-type *TP53* resulted in enhanced tumor cell proliferation. HCC patients bearing PIN1 expression with wild-type *TP53* were predicted to demonstrate favorable relapse-free survival. Our results suggest that PIN1 plays a role in cancer cell proliferation, migration and invasion in a different manner according to the *TP53* gene mutation status in HCC. In particular, interaction of PIN1 with mutant *TP53* can act as a tumor promoter and increase its oncogenic activities in HCC.

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most prevalent human cancer and the second common cause of cancer-related death worldwide (1). Although considerable advances have been made in clinical diagnosis and management of HCC, it is still associated with a high rate of mortality and poor prognosis (2). *TP53* is a tumor suppressor gene that plays important roles in cellular stress response and restrains cancer initiation and progression. *TP53* mutations change TP53 protein from a tumor suppressor into an oncogene (3). *TP53* mutations are among the most frequent genetic alterations in human cancer, including HCC (4,5). *TP53* mutations primarily occur in the DNA binding domain, resulting in disruption of tumor suppressor activity due to the inability to recognize wild-type *TP53* consensus sequences. *TP53* mutations also acquire new oncogenic functions through a gain of function (3,4,6). TP53 protein has been detected immunohistochemically in cancer cells by virtue of its high accumulation in cell nuclei and is regarded a highly specific indicator of *TP53* gene mutation (7).

Phosphorylation of proteins on serine/threonine residues that precede proline (pSer/Thr-Pro) is a main signaling mechanism controlling cell cycle regulation, differentiation and proliferation. Peptidyl-prolyl isomerase PIN1 can change the conformation of phosphoproteins and modulate the function and stability of proteins (8). Accumulating evidence has demonstrated that PIN1 is overexpressed in various human cancers and plays a critical role in the transformation of epithelial cells by activating multiple oncogenic pathways (9,10). In contrast, several studies have shown that decreased levels of

PIN1 result in cellular transformation, and restoration of PIN1 can attenuate the growth of tumor cells (8,11,12). These contradictory reports regarding the function of PIN1 in oncogenesis suggest that PIN1 can either function as a conditional tumor promoter or suppressor. Among the many documented targets of PIN1-mediated prolyl-isomerization that regulate cell fate, TP53 represents the most relevant one and is frequently deregulated in cancer (8,10,13). A recent study has shown that PIN1 conveys oncogenic signals in concert with mutant TP53 protein to promote aggressiveness in breast cancer cells, and concomitant high PIN1 expression and *TP53* mutation have been proposed as an independent prognostic factor of poor clinical outcome in breast cancer patients (14). Based on these observations, we hypothesized that PIN1 acts in a different manner according to the *TP53* gene mutation status in HCC.

In the present study, we examined: i) the relationship between PIN1 and TP53 protein expression in surgical specimens of human HCC; ii) the relationship between PIN1 expression and *TP53* mutation; iii) whether PIN1 silencing by small interfering RNA (siRNA) differently affects cell growth, migration, and invasion in HCC cells according to *TP53* mutation status and; iv) the association of PIN1 expression and *TP53* gene mutation status with clinical outcome.

Materials and methods

Materials. The present study protocol was approved by the Institutional Review Board of Chonbuk National University Hospital. Surgical specimens of 119 formalin-fixed, paraffin-embedded HCC obtained from the Surgical Pathology Archives of Chonbuk National University Hospital between 1998 and 2009 were analyzed in the present study. Patients were 25-74 years in age (mean age, 55.9) and consisted of 103 males and 16 females. A total of 91 cases were positive for hepatitis B virus surface antigen, 6 were positive for anti-hepatitis C virus antibody, 10 were alcohol-related and 12 had an unknown etiology. Overall survival was calculated from the date of surgery to the date of death or the final follow-up visit. Follow-up intervals ranged from 1-194 months. To determine whether PIN1 expression is associated with *TP53* mutation, we examined PIN1 protein levels in 5 HCC cell lines by western blotting. The human HCC cell lines HLE, HLF and Huh-7 containing mutant *TP53* (15) were purchased from the Health Science Research Resources Bank (Osaka, Japan). The HepG2 cell line (wild-type *TP53*) was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). We also used a sarcomatoid HCC cell line, designated as SH-J1 (wild-type *TP53*) (15). HCC cell lines were cultured according to the recommendations of the cell banks.

Immunohistochemical staining and scoring. Immunohistochemical staining for PIN1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and TP53 (Novocastra, Newcastle, UK) was performed by a polymer intense detection system using the Bond-Max Automatic stainer (Leica Bond, Newcastle upon Tyne, UK) as previously described (16). After deparaffinization, the tissue sections were heated in a microwave oven in Target Retrieval Solution (Dako, Glostrup, Denmark) for 12 min. The samples subjected to immunostaining were rated according to a score calculated by multiplying the area score by the intensity score of the staining. The area of staining was scored as follows: 0 (<10% of the cancer cells), 1 (10-29%), 2 (30-59%), or 3 (≥60%). The intensity of the cell nuclear staining was scored as 0 (none), 1 (weak), 2 (moderate), and 3 (strong). The combined score obtained by summing the scores was used for further analysis. If the score was ≥3, the tumor was considered positive; otherwise, the tumor was considered negative.

TA-cloning and DNA sequencing for TP53 gene. TA-cloning and DNA sequencing of the *TP53* gene were performed as previously described (15). The RNeasy Plus Micro kit (Qiagen, Hilden, Germany) was used according to the manufacturer's protocol for extraction of total RNA from 10 mg of frozen HCC tissue. Reverse transcription was performed using avian myeloblastosis virus reverse transcriptase (CosmoGenetech, Seoul, Korea) with an oligo(dT) primer supplied by the RT PreMix kit. The primer set for amplification of a human *TP53* cds was designed according to GenBank NM_000546, using forward primer, 5'-ATGGAGGAGCCGCAGTCAGATC CTAGCGTCGAG-3' and reverse primer, 5'-TCAGTCTGAGT CAGGCCCTTTTCTGTCTTGAA-3'. PCR conditions were 95˚C for 45 sec, 60˚C for 45 sec, and 72˚C for 90 sec for 35 cycles using LaboPass Pfu polymerase (CosmoGenetech). PCR products of human *TP53* were purified using a LaboPass PCR purification kit (CosmoGenetech) and cloned into a pCR2.1 vector (Invitrogen, Carlsbad, CA, USA). We obtained 5-18 clones for each individual sample and attempted to sequence as many clones as possible using a BigDye Terminator Cycle Sequencing Ready Reaction kit with an ABI PRISM 3730xl Genetic Analyzer (both from Applied Biosystems, Foster City, CA, USA).

Small interfering RNA (siRNA) transfection. For siRNA transfection, PIN1 siRNA and negative control siRNA duplexes were synthesized by Bioneer Corporation (Daejeon, Korea). The PIN1 duplex had the forward and reverse sequences: 5'-CCAUUUGAAGACGCCUCGU-3' and 5'-ACGAGGCGU CUUCAAAUGG-3', respectively; and the negative control duplex specific had the forward and reverse sequences: 5'-CCU ACGCCACCAAUUUCGU-3' and 5'-ACGAAAUUGGUGGC GUAGG-3'. The specific PIN1 siRNA or negative control siRNA was transfected using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. All experiments were performed at 48 h after transfection.

Enzymatic assay for PIN1. The level of PIN1 activity was determined using the SensoLyte® Green PIN1 Activity Assay kit *Fluorimetric* (AnaSpec, Inc., Fremont, CA, USA), according to the manufacturer's protocols. Briefly, PIN1 substrate solution was added to HCC cell extracts and incubated. Then, the fluorescein signal was read using a Multi-Mode Microplate Reader System (Perkin-Elmer, Waltham, MA, USA) at excitation and emission wavelengths of 490 and 520 nm, respectively.

Western blotting. Western blotting was performed as previously described (17). Briefly, the cells were collected, and pellets were lysed with PRO-PREP protein extraction solution (iNtRON Biotechnology, Inc., Seoul, Korea) containing 1X phosphatase inhibitor cocktails 2 and 3 (Sigma, St. Louis, MO, USA). The

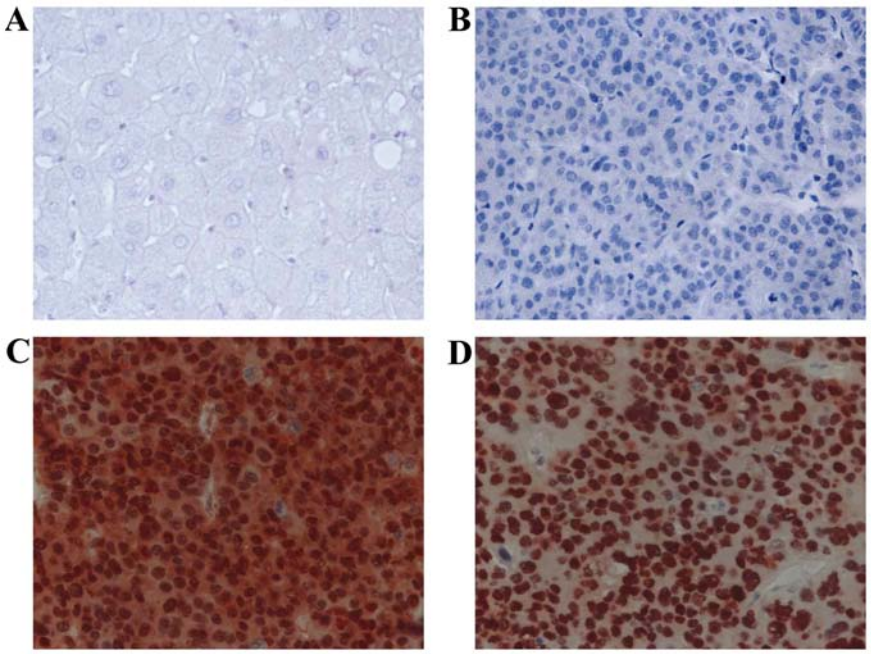

Figure 1. Immunohistochemical staining for PIN1 and TP53 protein. (A) Non-malignant hepatocytes showed no immunoreactivity for PIN1. (B) No immunoreactivity for PIN1 in HCC cells. (C) Both nuclear and cytoplasmic immunoreactivity for PIN1 in HCC cells. (D) The expression of TP53 was mainly localized in the nuclei of HCC cells.

proteins were separated by 10% SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific, Scotts Valley, CA, USA), and probed with primary antibodies for PIN1 (Santa Cruz Biotechnology), TP53 (Novocastra) and β-actin (Sigma).

Cell proliferation assay. The cell proliferation ability of PIN1 was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma). Post-transfection, the HepG2 or HLE cells were re-seeded in 96-well plates at 5 or $3x10^3$ cells/well and incubated for different times (24, 48 and 72 h). The absorbance of all samples was measured at 560 nm.

In vitro migration and invasion assays. A 24-Transwell migration assay (Corning Life Sciences, Acton, MA, USA) was performed to evaluate the migration ability of the cells. The invasion assay was performed using the Transwell bioCoat Matrigel Invasion chamber (BD Biosciences). The cells that migrated to or invaded the lower surface of the filter were counted in 5 microscopic fields (magnification, x100)/well.

Statistical analysis. To evaluate the values between the groups, Pearson's Chi-square test and Student's t-test were used. P-values <0.05 were considered to be statistically significant. All experiments were repeated a minimum of 3 times, and representative data are presented. Survival analyses were performed using the Kaplan-Meier method, and differences in survival between different groups were determined by the log-rank test.

Results

Relationship between PIN1 and TP53 protein expression. In HCC cells, PIN1 expression was predominantly localized in the nucleus. Various tumor cells showed both nuclear and cytoplasmic expression. The expression of TP53 was also mainly localized in the nuclei of tumor cells. Adjacent benign hepatocytes showed minimal or no immunoreactivity for PIN1 and TP53 (Fig. 1). Expression of PIN1 and TP53 was observed in 77 of 119 (64.7%) and 57 of 119 (47.9%) HCC tissues, respectively. There was a significant correlation between expression of PIN1 and TP53 in HCC tissues (P=0.002) (Table IA). The expression levels of PIN1 and TP53 proteins were higher in the Huh-7, HLE and HLF cell lines containing mutant *TP53* than in the HepG2 and SH-J1 cells containing wild-type *TP53* (Fig. 2A). In contrast, the enzymatic activity of PIN1

Table I. Correlation between PIN1 and TP53.

A, Correlation between PIN1 and TP53 protein expression

		TP53 expression		
		Positive	Negative	P-value
PIN1 expression	Positive	45	32	0.002
	Negative	12	30	

B, Correlation between PIN1 expression and *TP53* mutation

-		*TP53* mutation		
		Mutation	Wild-type	P-value
PIN1 expression	Positive	19	8	0.03
	Negative	5	9	

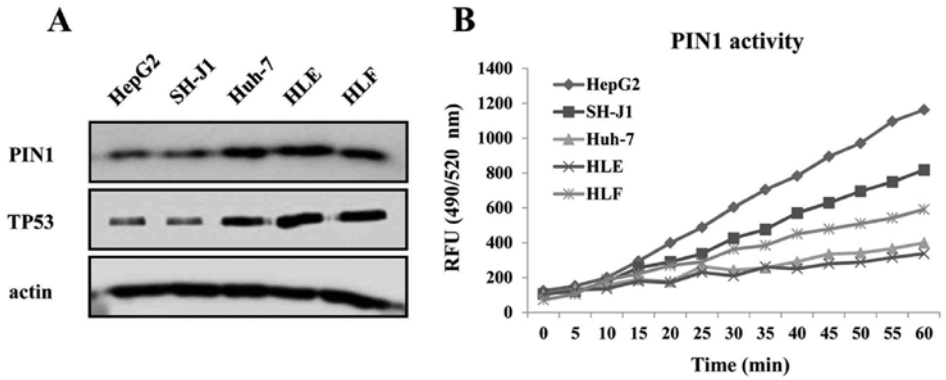

Figure 2. Western blot analysis and enzymatic activity of PIN1 in 5 different hepatocellular carcinoma cell lines. (A) The protein expression levels of PIN1 and TP53 protein were higher in Huh-7, HLE and HLF cell lines than in HepG2 and SH-J1 cells. (B) In contrast, the enzymatic activity of PIN1 was higher in the HepG2 and SH-J1 cells compared to that of HCC cell lines containing mutant *TP53*.

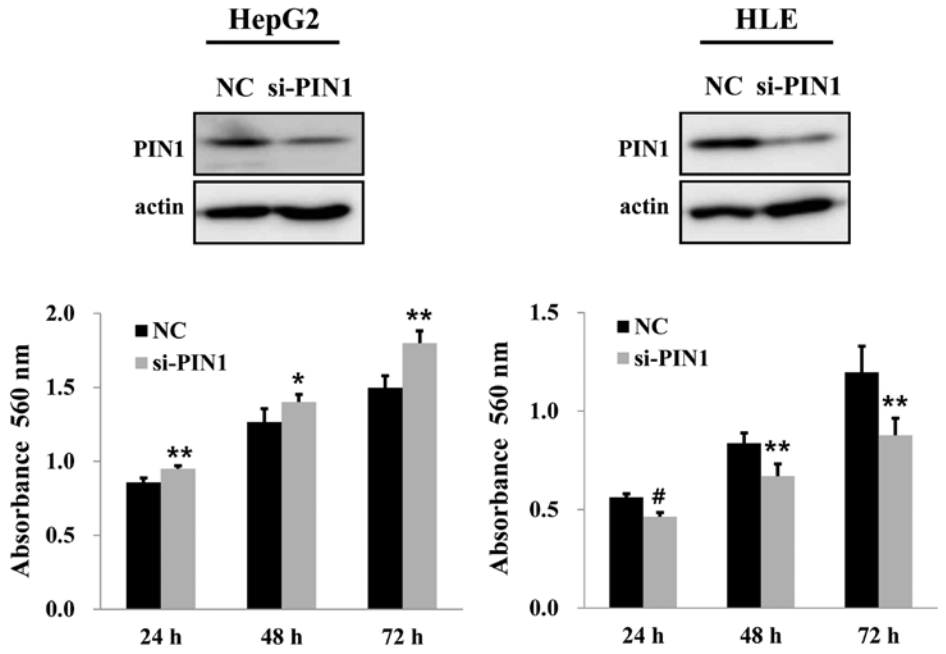

Figure 3. The effects of PIN1 silencing on HCC cell growth. PIN1 silencing in HepG2 cells increased the cell growth compared to that of the control. On the contrary, silencing of PIN1 resulted in significant decrease of cell growth in HLE cells (*P<0.05, **P<0.005, $^{\#}$P<0.001).

was higher in the HepG2 and SH-J1 cells compared to that of HCC cell lines containing mutant *TP53* (Fig. 2B).

Relationship between PIN1 expression and TP53 mutation. Positive immunostaining of TP53 is considered to be indicative of mutation or overexpression in response to cellular stress. PIN1 expression correlated with TP53 expression in our immunohistochemical study. To verify and confirm these immunohistochemical observations, we examined the relationship between PIN1 expression and *TP53* mutation in HCC tissues using *TP53* DNA sequencing. Forty-three frozen tissues were selected based on matching with the paraffin-embedded specimens using the immunohistochemical study. Of the 43 HCC frozen tissues, 26 had a mutation in the *TP53* gene. The majority of mutations were single-nucleotide substitutions (point mutations). Only 2 HCC tissues showed an insertion mutation. The mutations in *TP53* occurred predominantly in the hot-spot region (exon 5-8) [29 of 64) (45.3%) of *TP53* mutations]. Twenty-four HCCs bearing a *TP53* point mutation (missense mutation) were included in the analysis of the relationship between PIN1 expression and *TP53* mutation, since strong diffuse nuclear staining for TP53 antibody correlates with a missense mutation (4,6). In agreement with results of immunohistochemistry, the expression of PIN1 was significantly correlated with *TP53* mutation in HCC tissues (P=0.03) (Table IB). These results indicate that there is a good correlation between immunohistochemical expression and DNA sequencing of *TP53* in determining the relationship between PIN1 expression and *TP53* mutation in HCC tissues.

Effects of PIN1 silencing on cell proliferation, migration and invasion. PIN1 silencing by PIN1 siRNA in HepG2 cells containing wild-type *TP53* increased the cell growth compared to that of the control. On the contrary, silencing of PIN1 resulted in a significant decrease in cell growth in HLE cells with mutant *TP53* (Fig. 3). Silencing of PIN1 in HepG2

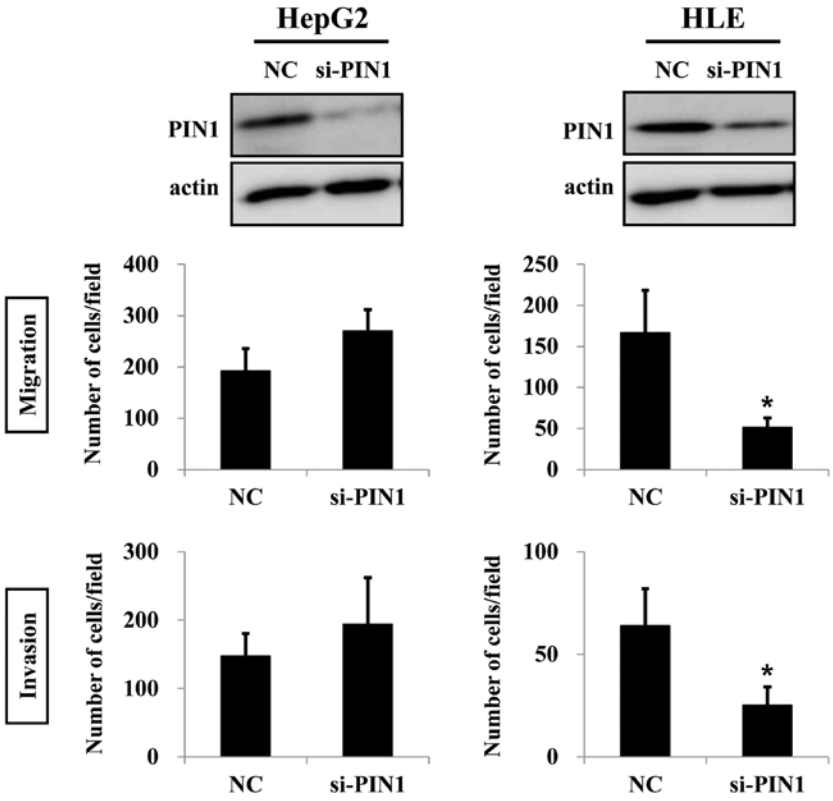

Figure 4. *In vitro* cell migration and invasion assay. PIN1 silencing effectively suppressed the migration and invasion of HLE cells. On the contrary, PIN1 silencing in HepG2 cells slightly increased cell migration and invasion (*P<0.05).

cells caused increases in migration and invasion. However, these results were not statistically significant. On the contrary, silencing of PIN1 significantly suppressed migration and invasion of HLE cells (Fig. 4).

Clinical relevance of PIN1 expression and TP53 mutation. We next examined the association of PIN1 expression and *TP53* gene mutation status with clinical outcome in 43 *TP53* DNA-sequenced HCC patients. The mean overall and relapse-free survival durations of patients were 71.2 and 65.6 months, respectively, for PIN1-positive HCC and 73.5 and 48.4 months for PIN1-negative HCC. PIN1 expression was associated with favorable relapse-free survival (RFS) independent of *TP53* mutation status (P=0.03). When we determined the RFS using a putative combination of PIN1 expression with *TP53* mutation status, we found that RFS was significantly longer in patients with HCCs bearing wild-type *TP53* and PIN1 expression compared to that in PIN1-negative HCC patients, according to Kaplan-Meier survival analysis (P=0.018) (Fig. 5). However, we did not find a significant effect on overall survival (OS) or RFS in patients with HCCs bearing mutant *TP53* according to PIN1 expression status.

Discussion

Frequent high expression of PIN1 in various human cancers strongly implicates PIN1 in carcinogenesis of many different cancer types, including HCC (8-10,18-21). PIN1 is critically involved in hepatocarcinogenesis via accumulation of β-catenin and cyclin D (19), or interaction with HBx in hepatitis B virus-related HCC (20). PIN1 also facilitates NF-κB activation and promotes tumor progression in HCC (21). However, it remains controversial whether PIN1 acts as a tumor promoter (10,14,18-20) or tumor suppressor (11,12,22). TP53 is one of the more than 50 critical regulatory proteins catalyzed by PIN1 (8,13). *TP53* mutation is also one of the most common genetic alterations in HCC and leads to the accumulation of mutant TP53 protein that endows oncogenic activities (5,7,23). It has been proposed that the PIN1/mutant TP53 axis promotes aggressiveness in breast cancer (14). Accordingly, the role of PIN1 expression in conjugation with *TP53* mutation status in human malignancy needs to be investigated. However, there have been no studies on the relationship between PIN1 expression with respect to *TP53* gene status and its roles in HCC.

The present study is the first to demonstrate the following: i) there is a significant correlation between immunohistochemical expression of PIN1 and TP53 protein in HCC; ii) expression of PIN1 is strongly associated with *TP53* mutation in HCC; iii) PIN1 and TP53 expression in *TP53* mutant HCC cell lines is higher compared to that in *TP53* wild-type HCC cell lines; in contrast, the enzymatic activity of PIN1 is higher in HCC cells with wild-type *TP53*; iv) PIN1 silencing effectively reduces tumor cell proliferation, but also cell migration and invasion capacity in HLE cells containing mutant *TP53* gene. In contrast to PIN1 silencing in HLE cells, PIN1 silencing in HepG2 cells containing functional wild-type *TP53* yields different results: PIN1 silencing enhances tumor cell proliferation, migration and invasion; and v) patients bearing wild-type *TP53* with PIN1 expression show favorable relapse-free survival. These

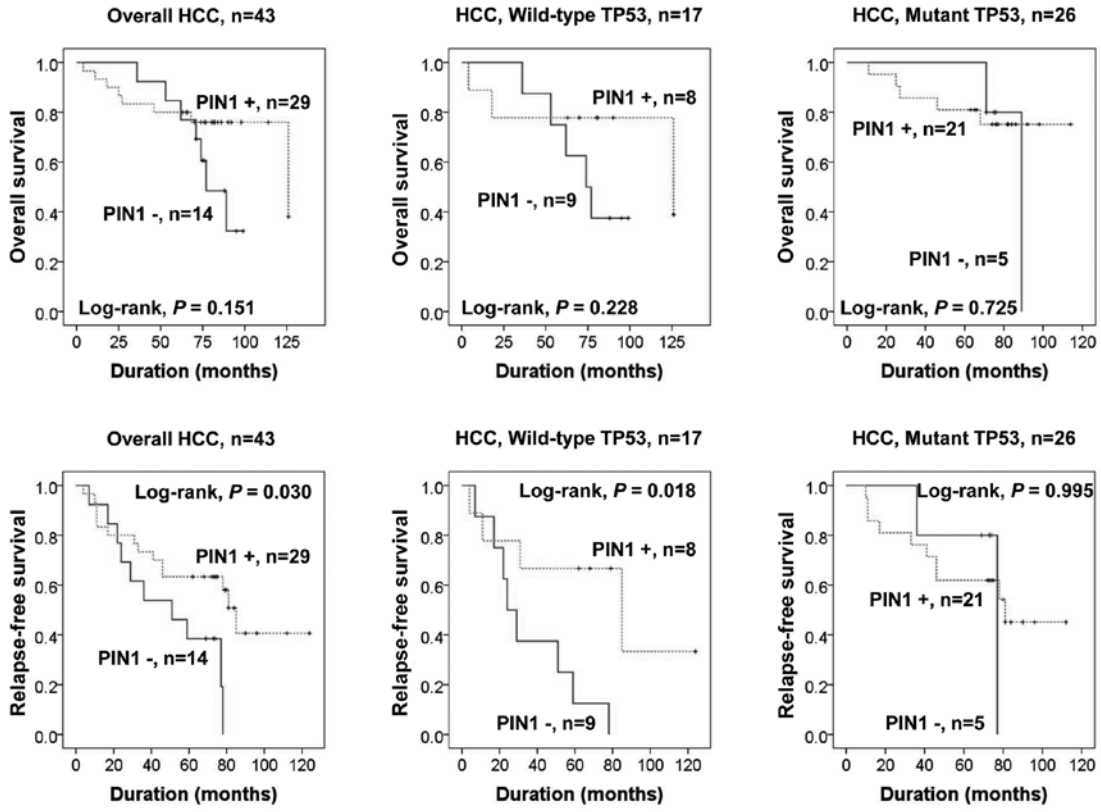

Figure 5. PIN1 expression was associated with favorable relapse-free survival independent of *TP53* mutation status. RFS was significantly higher in patients with HCCs bearing wild-type *TP53* with PIN1 expression than in PIN1-negative HCC patients.

findings strongly suggest that a functional interaction between PIN1 and TP53 produces different biologic outputs in tumor cell growth, migration and invasion depending on *TP53* gene mutation status.

TP53 is a tumor suppressor that initiates cell cycle arrest, apoptosis, and senescence in response to cellular stress (3,4,6). The isomerization of TP53 protein by PIN1 results in an alteration of protein structure/or function, which is often coupled to the stabilization of the TP53 protein (8-10). We found a correlation between expression of PIN1 and TP53 protein or *TP53* mutation in HCC. These observations are consistent with those of previous studies, which have reported a correlation between high expression level of PIN1 and high levels of TP53 protein in non-small cell lung and esophageal cancer (24,25). Since PIN1 has been shown to stabilize wild-type TP53 (8-10), these observations suggest a possible role of PIN1 in stabilization of mutant TP53. In the present study, transfection with PIN1 siRNA in HepG2 cells containing wild-type *TP53* caused an increase in cell growth; these findings are consistent with the physiologic role of PIN1, which induces cell cycle arrest and growth inhibition through interaction with wild-type TP53. This observation suggests that PIN1 functions as a tumor suppressor in conditions of intact TP53 signaling. In contrast, silencing of PIN1 resulted in a significant decrease in cell growth in HLE cells. This observation is in agreement with results from previous studies showing that RNAi-mediated PIN1 suppression inhibits HCC cell proliferation using PLC/PRF/5 and Huh-7 cell lines, which have a mutant *TP53* gene (18,21). It is noteworthy, that the underexpression of PIN1 is frequently seen in human cancers where the *TP53* mutation is rare, including kidney and skin cancer (9,12,22). PIN1 negatively influences the growth of human clear cell renal cell carcinoma (ccRCC), and this suppressive ability may be dependent on the presence of functional TP53 (12). Collectively, these observations support the notion that PIN1 can either function as a tumor promoter or suppressor depending on the genetic context. In particular, interaction of PIN1 with mutant TP53 promotes its oncogenic activities in *TP53* mutant HCC. However, PIN1 may have a tumor inhibitory role in the presence of functional wild-type TP53.

Several studies have indicated that, in addition to the role of PIN1 in oncogenesis, its expression is also associated with cancer cell migration and invasion. Downregulation of PIN1 expression significantly reduces tumor cell migration and invasion in various types of cancers occurring in the breast (14), lung (26) and prostate (27), where the *TP53* mutation is frequent. Consistent with these observations, we found that PIN1 silencing effectively suppressed the migration and invasion in HLE cells containing mutant *TP53* gene. Similarly, Girardini *et al* have demonstrated that RNAi-mediated knockdown of either mutant TP53 or PIN1 significantly attenuated cell migration and invasion of MDA-MB-231 breast cancer cells containing mutant *TP53* (14). Notably, in contrast to PIN1 silencing in HLE cells, PIN1 silencing in HepG2 cells containing wild-type *TP53* gene yielded slightly increased migration and invasion. These findings suggest that an interaction between PIN1 and functional TP53 results in cellular

effects opposite to those occurring in the presence of mutant TP53. High expression of PIN1 is correlated with poor prognosis in several types of cancers, including HCC (21,24-27). However, previous studies did not perform *TP53* gene mutation analysis, or details of the *TP53* gene status were not provided. The biological behavior of PIN1 according to the context of *TP53* gene in HCC remains unclear. We found that RFS was significantly longer in patients with HCC wild-type *TP53* with PIN1 expression. Similar to our result, Lill *et al* have demonstrated that high expression of PIN1 is a good prognostic factor in patients with Merkel cell carcinoma, which shows infrequent *TP53* mutation (22,28). Girardini *et al* have reported that OS is significantly decreased in patients with breast cancer expressing high levels of PIN1 and mutant *TP53* compared to that of patients with low PIN1 expression and mutant or wild-type *TP53* (14). However, PIN1 expression in combination with *TP53* mutation was not found to be a poor prognostic factor in the present study. This discrepancy in the clinical relevance of PIN1 may be partly explained by differences in tumor type and an insufficient number of patients in the present study. Additional investigations with a larger population of HCC patients with simultaneous assessment of the *TP53* gene and other cellular partner genes of PIN1 are necessary to determine the combination of PIN1 expression and mutant *TP53* gene that serves as a prognostic and predictive tool.

In conclusion, our findings strongly suggest that the PIN1 can exert a conditional tumor promoter or suppressor role depending on the *TP53* gene mutation status in HCC. Our results also support the need to evaluate the status of the *TP53* gene in the development of therapeutic approaches for targeting PIN1 in HCC patients.

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